Paeoniflorin protects PC12 cells from oxygen-glucose deprivation/reoxygenation-induced injury via activating JAK2/STAT3 signaling

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Abstract. Ischemic stroke is the most common type of stroke, and it has become a major health issue as it is characterized by high mortality and morbidity rates. Paeoniflorin (PF) is a natural compound and the main active ingredient of Radix Paeoniae. The aim of the present study was to investigate the role of PF in oxygen-glucose deprivation/reoxygenation (OGD/R)-induced injury of PC12 cells and its association with the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway. An *in vitro* model of OGD/R injury was established in PC12 cells. Subsequently, Cell Counting Kit-8 assay and ELISA were used to evaluate cell viability and the secretion of inflammatory factors, respectively, in PC12 cells subjected to OGD/R and treated with PF. The levels of oxidative stress indicators and inflammatory factors were measured using corresponding commercial kits. In addition, the apoptosis rate of PC12 cells subjected to OGD/R and treated with PF was determined by flow cytometry, and the expression of apoptosis-related proteins was analyzed by western blotting. Additionally, the expression levels of JAK2/STAT3 pathway-related proteins were also evaluated. The cell viability, levels of oxidative stress, inflammation and apoptosis were also measured in OGD/R-induced PC12 cell injury models following co-treatment of cells with PF and FLLL32, a specific inhibitor of JAK2/STAT3 signaling. Cell viability was reduced, while oxidative stress and inflammation were increased after OGD/R-induced injury. However, the treatment of cells with PF significantly enhanced cell viability, and alleviated oxidative stress, inflammation and apoptosis of OGD/R-treated PC12 cells. Furthermore, PF activated the JAK2/STAT3 signaling pathway. Following FLLL32 intervention, the effects of PF on oxidative stress, inflammation and apoptosis of OGD/R-treated PC12 cells were reversed. In conclusion, the findings of the present study suggested that PF may protect PC12 cells from OGD/R-induced injury via activating the JAK2/STAT3 signaling pathway, thus providing novel insight into the mechanism through which PF may alleviate ischemic stroke and indicating a potential strategy for ischemic stroke treatment.

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Introduction

Ischemic stroke is a major type of stroke that is characterized by high mortality and morbidity rates (1). The oxygen and glucose deprivation (OGD) that results from complete or partial blockade of arterial blood supply to the brain is considered as the leading cause for the occurrence of ischemic stroke (2). Ischemia-reperfusion (I/R) injury, which is defined as the restoration of blood supply after a given duration of ischemia, is a common characteristic of ischemic stroke (3). Although the incidence of ischemic stroke is high, effective treatment strategies for this disease are still lacking.

Paeoniflorin (PF; Fig. 1A), a natural compound, is the main active ingredient of *Radix Paeoniae* (4). A previous study demonstrated that PF protected HT-22 cells from H_2O_2-induced oxidative injury via regulating the expression of microRNA(miR)-135a(5). In addition, 6'-O-galloylpaeoniflorin, the galloylated derivative of PF isolated from peony root, attenuated neuroinflammation and oxidative stress in a cerebral I/R injury rat model via activating the PI3K/AKT/nuclear factor erythroid-2-like 2 signaling pathway (6). Compelling evidence has indicated that PF combined with β-ecdysterone protected PC12 cells against neurotoxicity triggered by rotenone (7). Additionally, 6-hydroxydopamine-induced PC12 cell apoptosis was suppressed by PF via blocking the reactive oxygen species-induced protein kinase C δ/NF-κB signaling pathway (8). Another study revealed that the function of PF in preventing mitochondrial dysfunction may alleviate cytotoxicity in glutamate-induced PC12 cells (9). Importantly, it has also been reported that the natural neuroprotector PF can inhibit a number of pro- and anti-inflammatory signals in differentiated PC12 cells (10).

It has been suggested that Janus kinase 2 (JAK2), a crucial factor involved signaling through a variety of cytokine receptors, phosphorylates signal transducer and activator of transcription 3 (STAT3) upon activation (11). A

previous study indicated a strong association between the phosphorylation/activation of the JAK2/STAT3 pathway and a neuroprotection-related signaling pathway (12). Blocking the JAK2/STAT3 signaling pathway by PF may protect the kidneys of diabetic rats (13). Furthermore, the melatonin-mediated regulation of the miR-26a-5p/neuron-restrictive silencer factor and JAK2/STAT3 pathways may attenuate cerebral I/R injury via alleviating inflammation and oxidative stress (14). In addition, the activation of the JAK2/STAT3 pathway by Src homology 2B adaptor protein 1 protected PC12 cells from oxygen-glucose deprivation/reoxygenation (OGD/R)-induced apoptosis (15). Therefore, it was hypothesized that PF may play a significant role against OGD/R-induced PC12 cell injury via regulating the JAK2/STAT3 pathway.

The aim of the present study was to investigate the function of PF in OGD/R-induced PC12 cell injury and the role of the JAK2/STAT3 signaling pathway in this process, in order to elucidate whether PF may be considered as a promising candidate for the treatment of ischemic stroke.

Materials and methods

PC12 cell culture and treatment. The rat pheochromocytoma cell line, PC12, was obtained from the American Type Cell Culture Collection. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and streptomycin at 37˚C in a humidified incubator containing 5% CO_2. PF (purity, >98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). PF was dissolved in DMSO (Sigma-Aldrich; Merck KGaA) to a final concentration of 200 μM. A total of 100 μl PF (200 μM) was diluted to 100, 50 and 25 μM PF by adding into 100, 300 and 700 μl DMEM, respectively. The final concentration of DMSO in the cultures was <1%. The cells were detached and re-seeded into six-well plates ($1x10^6$ cells per well) for the subsequent experiments. Finally, the cells were left untreated or were pre-treated with various concentrations of PF for 24 h, followed by treatment with FLLL32, a specific inhibitor of JAK2/STAT3 signaling, at a dose of 25 μM for an additional 24 h.

Establishment of OGD/R injury model. PC12 cells were cultured in Earle's Balanced Salt Solution (Sigma-Aldrich; Merck KGaA) without glucose to induce cell ischemia. The cells were maintained in a three-gas incubator containing 94% N_2, 5% CO_2 and 1% O_2 at 37˚C for 4 h. Subsequently, the medium was discarded and replaced with normal medium supplemented with 10% FBS, and cells were reoxygenated in a normal atmosphere for an additional 24 h for the establishment of the reperfusion model.

Cell Counting Kit-8 (CCK-8) assay. To determine PC12 cell viability, the cells were seeded into a 96-well plate at a density of $3x10^4$ cells per 100 μl medium in each well. Following incubation for 24 h, 10 μl CCK-8 reagent was added into each well, and the plate was incubated at 37˚C for an additional 4 h. The absorbance of each well was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.).

Determination of oxidative stress. Briefly, $2x10^5$ PC12 cells/well were seeded into 96-well plates. The activity of lactate dehydrogenase (LDH; cat. no. A020-2-2), myeloperoxidase (MPO; cat. no. A044-1-1) and superoxide dismutase (SOD; cat. no. A001-3-2) was determined using the corresponding kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturers' instructions. Subsequently, the absorbance was determined at 450 nm (LDH and SOD) or 460 nm (MPO) using a microplate reader (Bio-Rad Laboratories, Inc.).

Determination of the levels of inflammatory factors. ELISA kits were used to measure the levels of TNF-α (cat. no. F16960), IL-6 (cat. no. F15870) and IL-10 (cat. no. F15900) in the cell culture medium. The cells were first treated with 500 μl ice-cold carbonate buffer (100 mM Na_2CO_3, 50 mM NaCl, pH 11.5) supplemented with protease inhibitors, and were then dissociated using an ultrasonic cell disruption system (Ningbo Haishu Kesheng Ultrasonic Equipment Co., Ltd.). The mixture was centrifuged at 12,000 x g for 45 min at 4˚C. The supernatant was then collected, and the levels of the inflammatory factors were measured according to the manufacturer's instructions (Shanghai Xitang Biotechnology Co., Ltd.).

Flow cytometric analysis. Flow cytometry using the Annexin V-FITC apoptosis kit (Beyotime Institute of Biotechnology) was performed to determine cell apoptosis. Following a series of treatments as aforementioned, PC12 cells were collected by centrifugation (200 x g; 10 min; room temperature), then washed twice by ice-cold PBS, resuspended in 195 μl pre-chilled 1X Annexin V binding buffer. Subsequently, cells were doubled-stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) for 15 min in the dark at room temperature according to the manufacturer's instructions. Subsequently, cell apoptosis of each sample was assessed by flow cytometer (BD Accuri™ C6; BD Biosciences). The data were analyzed using FlowJo software (version 7.6.1; FlowJo LLC). Annexin V and PI single-stained positive cells were used to regulate compensation (as control). The sum of apoptosis rate in right upper quadrant (Q2, late apoptotic cells) and right lower quadrant (Q3, early apoptotic cells) were considered as the cell apoptosis rate. The apoptotic cells were expressed as a percentage of the total number of cells.

Western blot analysis. Total proteins were extracted from PC12 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was determined by a BCA Protein Assay kit (Beyotime Institute of Biotechnology). The protein samples (40 μg/lane) were then separated by 10% SDS-PAGE and transferred onto a PVDF membrane (EMD Millipore). Subsequently, the membrane was blocked with 5% skimmed milk for 2 h at room temperature, washed with Tris-buffered saline containing 0.2% Tween-20 (TBST; Boster Biological Technology), and incubated with the primary antibodies at 4˚C overnight. After washing with TBST, the membrane was incubated with a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; cat. no. 7074S; Cell Signaling Technology, Inc.) or horse anti-mouse HRP-conjugated secondary antibody (1:3,000; cat. no. 7076S; Cell Signaling Technology, Inc.) at room temperature for 2 h. Finally, protein

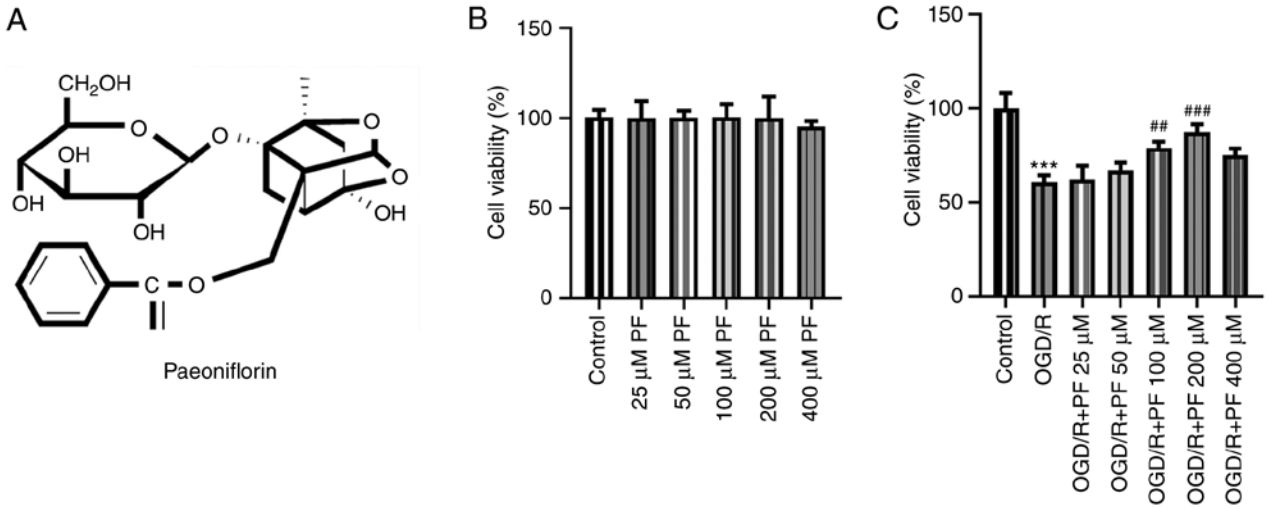

Figure 1. PF enhances the viability of OGD/R-treated PC12 cells. (A) Chemical structure of PF. (B) CCK-8 assay was used to determine the viability of PC12 cells treated with increasing doses of PF. (C) Following establishment of OGD/R-induced PC12 cell injury model, the apoptosis rate of cells treated with increasing doses of PF was determined using CCK-8 assay. ***P<0.001 vs. the control group; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the OGD/R group. PF, paeoniflorin; OGD/R, oxygen-glucose deprivation/reoxygenation; CCK-8, Cell Counting Kit-8.

bands were visualized using an enhanced chemiluminescence substrate (Pierce; Thermo Fisher Scientific, Inc.) on a chemiluminescence imaging equipment (Ultra-Lum, Inc.). The proteins bands were quantified using the ImageJ software (version 1.52r; National Institutes of Health). The gray value of the target protein was normalized to that of GAPDH. The following primary antibodies were used: Anti-Bax (cat. no. 14796S; 1:1,000), anti-cleaved caspase-9 (cat. no. 20750S; 1:1,000), anti-cleaved poly(adenosine diphosphate-ribose) polymerase (PARP) (cat. no. 9185S; 1:1,000), anti-p-JAK2 (cat. no. 3776S; 1:1,000), anti-JAK2 (cat. no. 3230T; 1:1,000), anti-p-STAT3 (cat. no. 9145S; 1:1,000), anti-STAT3 (cat. no. 4904T; 1:1,000) and anti-GAPDH (cat. no. 5174S; 1:1,000). All antibodies were obtained from Cell Signaling Technology, Inc., apart from anti-Bcl-2 (cat. no. sc-7382; 1:1,000), which was purchased from Santa Cruz Biotechnology, Inc.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 6.0; GraphPad Software, Inc.) and data are expressed as the mean ± SD. All experiments were performed three times. Statistical comparisons among multiple groups were analyzed using one-way ANOVA followed by a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

PF enhances the viability of PC12 cells subjected to OGD/R. The viability of PC12 cells was determined to evaluate the effect of different concentrations of PF. As shown in Fig. 1B, treatment of PC12 cells with 25, 50, 100 or 200 μM PF did not affect cell viability, whereas treatment with 400 μM PF decreased PC12 cell viability. After the establishment of the OGD/R cell model, the viability of PC12 cells was notably decreased compared with the control group, and it was gradually restored following treatment with increasing concentrations of PF (25, 50, 100 or 200 μM). This finding suggested that PF could increase the viability of OGD/R-treated PC12 cells (Fig. 1C). Since treatment with 400 μM PF promoted PC12 cell injury and exerted a weaker effect on OGD/R-treated PC12 cells compared with 200 μM PF, doses of 25-200 μM PF were selected for the subsequent experiments.

PF alleviates oxidative stress, inflammation and apoptosis in OGD/R-treated PC12 cells. To determine whether PF could mitigate the OGD/R-induced cell injury, the levels of oxidative stress indicators and inflammatory factors were determined. The levels of LDH and MPO were significantly increased, while those of SOD were markedly reduced by OGD/R. Treatment with increasing concentrations of PF reduced the levels of LDH and MPO, and increased those of SOD (Fig. 2A-C). Additionally, the ELISA results demonstrated that the levels of TNF-α and IL-6 were increased and those of IL-10 were decreased in the OGD/R group compared with the control group, while treatment with PF had the opposite effect (Fig. 2D-F). Overall, the aforementioned results suggested that PF could attenuate oxidative stress and inflammation in OGD/R-treated PC12 cells.

Apoptosis is considered as one of the most important cellular processes in OGD/R-induced injury. Therefore, the apoptosis of OGD/R-treated PC12 cells was determined. As shown in Fig. 3A and B, cell apoptosis was enhanced among OGD/R-treated PC12 cells; however, PF alleviated cell apoptosis in a dose-dependent manner. Furthermore, the expression of the pro-apoptotic proteins Bax, cleaved caspase-9 and cleaved PARP was notably upregulated, while that of the anti-apoptotic protein Bcl-2 was downregulated by OGD/R. These effects were reversed by increasing concentrations of PF (Fig. 3C). Collectively, these results revealed that PF attenuated oxidative stress, inflammation and apoptosis in OGD/R-treated PC12 cells.

PF activates JAK2/STAT3 signaling in OGD/R-treated PC12 cells. Subsequently, the possible association between PF and the JAK2/STAT3 signaling pathway was evaluated by western blot analysis. It was found that the protein expression levels

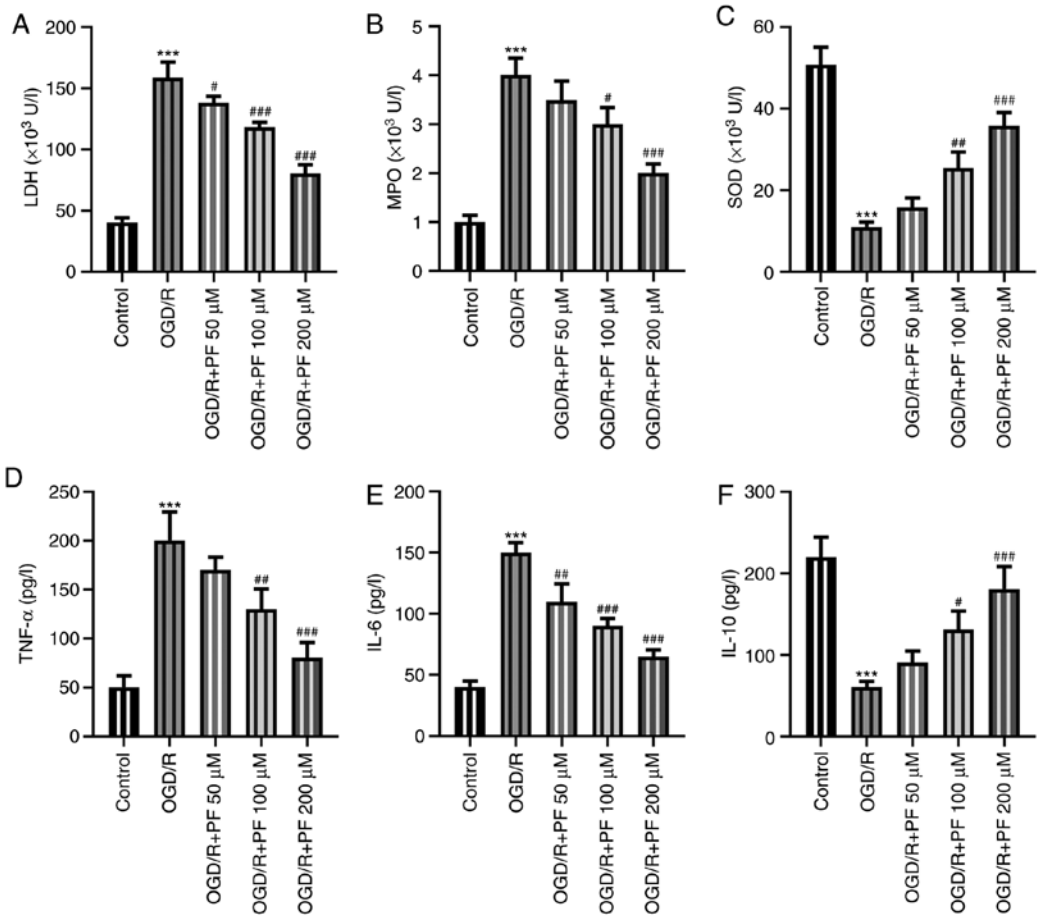

Figure 2. PF alleviates oxidative stress and inflammation in OGD/R-treated PC12 cells. Activity of (A) LDH, (B) MPO and (C) SOD in PC12 cells subjected to OGD/R and treated with PF was measured using commercial kits. ELISA was used to determine the secretion levels of the inflammatory factors (D) TNF-α (E) IL-6 and (F) IL-10. ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the OGD/R group. PF, paeoniflorin; OGD/R, oxygen-glucose deprivation/reoxygenation; LDH, lactate dehydrogenase; MPO myeloperoxidase; SOD, superoxide dismutase.

of phosphorylated (p)-JAK2 and p-STAT3 were significantly decreased in the OGD/R-treated group compared with the untreated group (Fig. 4). In addition, treatment with increasing doses of PF gradually enhanced the expression levels of p-JAK2 and p-STAT3 compared with the OGD/R group. These results suggested that the JAK2/STAT3 signaling pathway may be activated by PF. To better evaluate the effects of PF, the concentration of 200 μM was selected for the subsequent experiments.

FLLL32 abrogates the inhibitory effects of PF on oxidative stress, inflammation and apoptosis in OGD/R-treated PC12 cells. Subsequently, PC12 cells subjected to OGD/R were co-treated with the JAK2/STAT3 signaling inhibitor, FLLL32, after PF addition, to verify whether PF exerted its protective effects on these cells via the JAK2/STAT3 signaling pathway. As shown in Fig. 5A, the OGD/R-mediated reduced PC12 cell viability was restored by PF, while co-treatment with FLLL32 decreased the cell viability. The effect of PF on alleviating oxidative stress and inflammatory responses was partially counteracted by FLLL32 intervention (Fig. 5B-G). As regards cell apoptosis, PF suppressed the OGD/R-mediated PC12 cell apoptosis, which was further promoted by FLLL32 (Fig. 6A and B). Consistent with the previous findings, treatment with FLLL32 markedly reduced the expression levels of Bcl-2, which was accompanied by the upregulated expression of Bax, cleaved caspase-9 and cleaved PARP, compared with the OGD/R + PF group. The aforementioned findings verified that FLLL32 could abrogate the protective effects of PF on OGD/R-treated PC12 cells.

Discussion

In vivo and *in vitro* studies have suggested that PF has potent anti-inflammatory and immunosuppressive properties, and it can reduce pain, joint swelling, synovial hypertrophy, bone erosion and cartilage degradation in arthritic rats (16-18). In addition, PF was shown to regulate the immune responses and increase the survival rate of septic rats (19). Furthermore, PF exerted antioxidant and anti-apoptotic effects in the treatment of cholestatic liver injury (20). Therefore, the present study sought to determine whether PF could also attenuate inflammation, oxidative stress and apoptosis in the treatment of ischemic stroke.

Oxidative stress is widely involved in several pathophysiological processes, such as aging, inflammation and tumorigenesis (21). A study suggested that pre-treatment of PC12 cells with PF may enhance cell viability and decrease the release of LDH (4). This finding was consistent with the results of the present study. MPO is a critical inflammatory

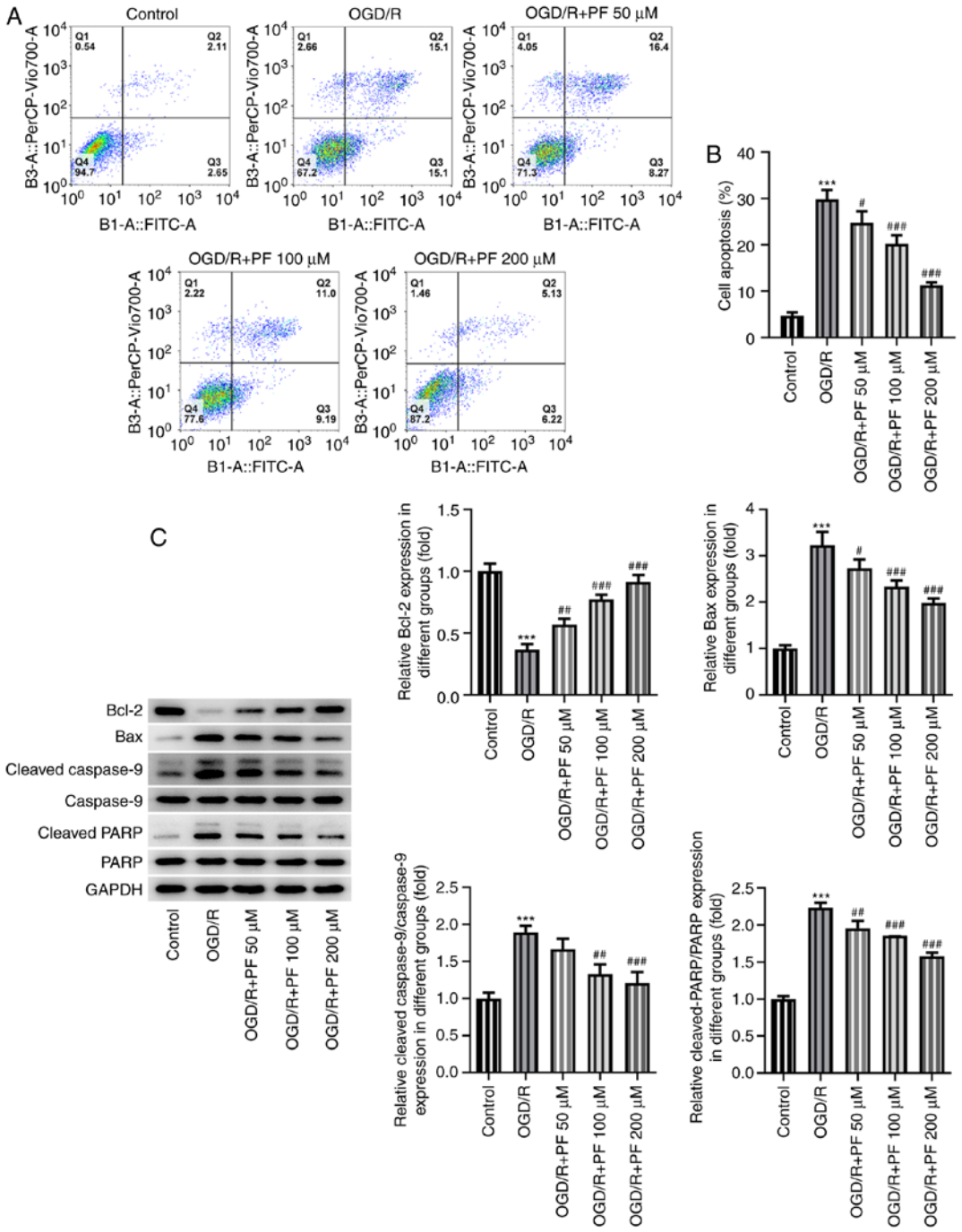

Figure 3. PF attenuates the apoptosis of OGD/R-treated PC12 cells. (A and B) Flow cytometry was used to evaluate the apoptosis of PC12 cells subjected to OGD/R and treated with PF. (C) Western blot analysis was used to determine the expression levels of apoptosis-related proteins in OGD/R-treated PC12 cells. ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the OGD/R group. PF, paeoniflorin; OGD/R, oxygen-glucose deprivation/reoxygenation.

enzyme and therapeutic target triggering both oxidative stress and neuroinflammation during the pathological process of cerebral I/R injury (22). MPO is often upregulated in various inflammatory cells, while its inhibition has been associated with the development of a relatively protective environment from brain damage in a murine model of stroke (23). Furthermore, SOD can directly affect the antioxidant capacity (24,25). Increased SOD activity was shown to enhance the protective mechanism against cerebral I/R injury in diabetic rats (26). Emerging evidence has suggested that PF can protect HT-22 cells from H_2O_2-induced oxidative injury via regulating the expression of miR-135a (5). The present study demonstrated that PF alleviated oxidative stress in OGD/R-treated PC12 cells.

Previous studies have reported that PF exerts anti-inflammatory effects in order to regulate cellular functions (27-30). In the present study, treatment with PF also reduced the production of inflammatory cytokines. Apoptosis is one of the most important pathophysiological effects of ischemic stroke, and the aberrant expression of apoptosis-related proteins has been considered to serve as indicator or marker of cell death (11,31). Other studies also supported the neuroprotective effects of PF, mediated by decreased Bax and increased Bcl-2 expression (4). Consistent with previous findings, in the present

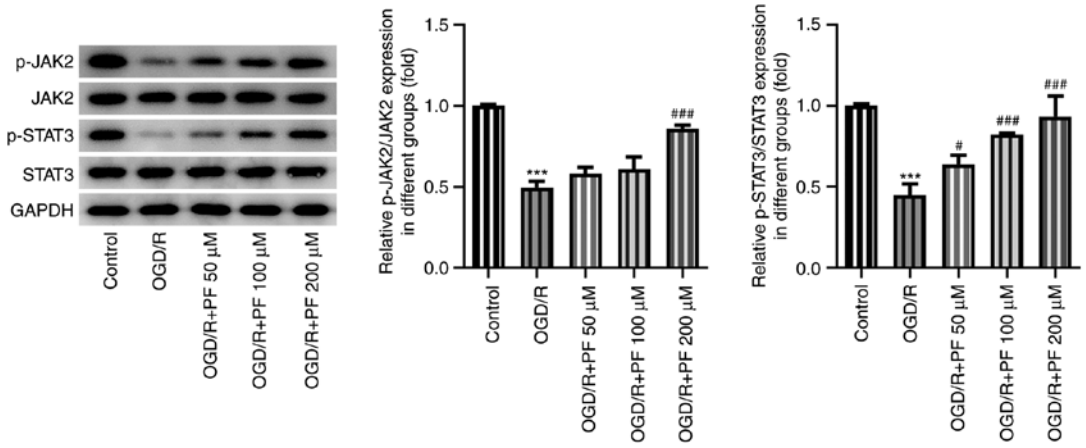

Figure 4. PF activates the JAK2/STAT3 signaling pathway in OGD/R-treated PC12 cells. The expression levels of the JAK2/STAT3 signaling-related proteins were detected by western blotting. ***P<0.001 vs. the control group; #P<0.05, ###P<0.001 vs. the OGD/R group. PF, paeoniflorin; OGD/R, oxygen-glucose deprivation/reoxygenation; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3.

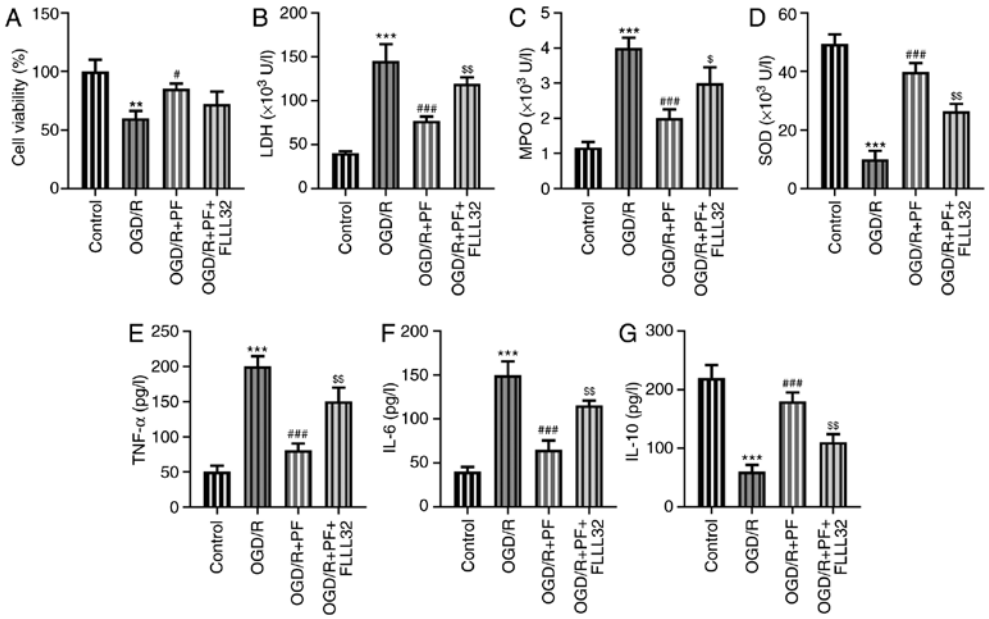

Figure 5. FLLL32, a specific inhibitor of Janus kinase 2/signal transducer and activator of transcription 3 signaling, abrogates the antioxidant and anti-inflammatory effects of PF on OGD/R-treated PC12 cells. (A) Cell viability was assessed using Cell Counting Kit-8 assay. The expression levels of the oxidative stress indicators (B) LDH, (C) MPO and (D) SOD were measured using commercial kits. The secretion levels of the inflammatory factors (E) TNF-α (F) IL-6 and (G) IL-10 in PC12 cells subjected to OGD/R and treated with PF and FLLL32 were determined using ELISA. **P<0.01 ***P<0.001 vs. the control group; #P<0.05, ###P<0.001 vs. the OGD/R group; $P<0.05, $$P<0.01 vs. the OGD/R + PF group. PF, paeoniflorin; OGD/R, oxygen-glucose deprivation/reoxygenation; LDH, lactate dehydrogenase; MPO myeloperoxidase; SOD, superoxide dismutase.

study PF promoted the down- and upregulation of pro- and anti-apoptotic proteins, respectively, in OGD/R-treated PC12 cells. Therefore, PF attenuated oxidative stress, inflammation and apoptosis in OGD/R-treated PC12 cells.

It has been reported that the JAK2/STAT3 signaling pathway serves as a potent effector in attenuating cell death and apoptosis (32). JAK2/STAT3 signaling has also been associated with the occurrence and progression of several inflammatory diseases, including arthritis, apical periodontitis and Alzheimer's disease (33-35). JAK2 is essential for signaling through a variety of key class I cytokine receptors, such as IL-3, IL-6, interferon-γ and leptin (36). The STAT transcription factors are crucial for cell fate. Among the members of the STAT family, STAT3 is involved in classic inflammatory diseases (37). A previous study demonstrated that the activation of the JAK2/STAT3 signaling pathway by different drugs protected mice from a series of pathological stress stimuli and I/R injury (38). In the present study, the expression levels of p-JAK2 and p-STAT3 were increased by PF in OGD/R-treated PC12 cells, suggesting that this signaling pathway may be activated by PF. Consistent with the findings of the present study, a previous study demonstrated that abolishment of the JAK2/STAT3 signaling pathway by a pharmacological inhibitor aggravated apoptosis and OGD/R injury (39).

Overall, the present study demonstrated that PF may protect PC12 cells from OGD/R-induced injury partly via activating JAK2/STAT3 signaling. Furthermore, the findings

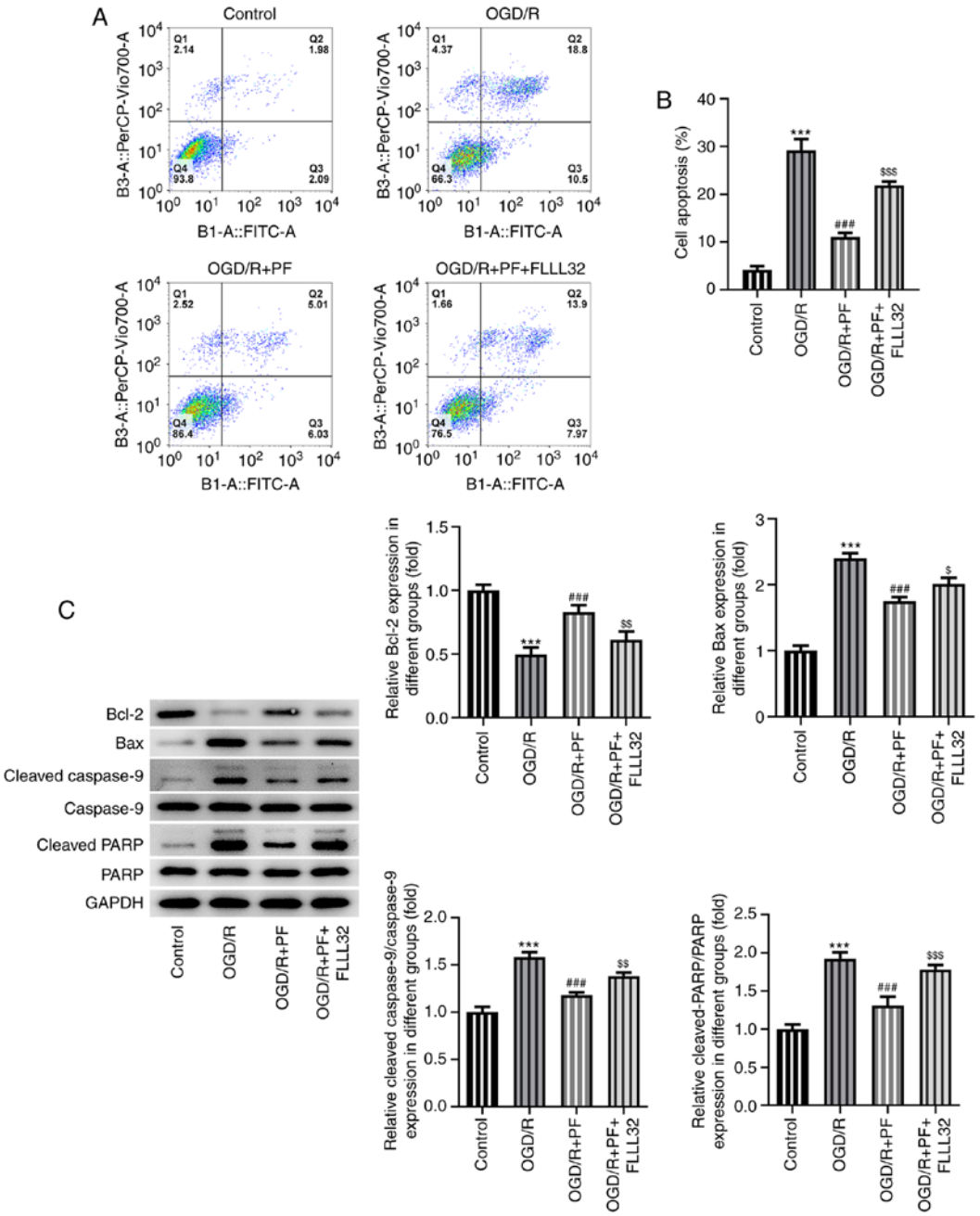

Figure 6. FLLL32, a specific inhibitor of Janus kinase 2/signal transducer and activator of transcription 3 signaling, attenuates the anti-apoptotic effects of PF on OGD/R-induced PC12 cells. (A and B) Flow cytometry was used to evaluate the apoptosis of PC12 cells subjected to OGD/R and co-treated with PF and FLLL32. (C) Western blot analysis was used to determine the expression levels of apoptosis-related proteins in PC12 cells subjected to OGD/R and treated with PF and FLLL32. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the OGD/R group; $^{\$\$}$P<0.01, $^{\$}$P<0.05, $^{\$\$\$}$P<0.001 vs. the OGD/R + PF group. PF, paeoniflorin; OGD/R, oxygen-glucose deprivation/reoxygenation; PARP, poly(adenosine diphosphate-ribose) polymerase.

of the present study may provide a better understating of the mechanism through which PF alleviates the adverse effects of ischemic stroke and indicate a promising approach to treating this disease. The lack of studies *in vivo* is a limitation of the present research. Based on the present findings, the effects of PF on the functional recovery of animals with cerebral I/R injury will be studied in a middle cerebral artery occlusion and reperfusion rat model. Additionally, whether PF can inhibit oxidative stress, inflammation and apoptosis and the regulatory effects of JAK2/STAT3 signaling and other potential pathways will be further investigated in the following experiments.

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Availability of materials and data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ and WY searched the literature, designed the experiments and performed the experiments. ZZ analyzed, interpreted the data and wrote the manuscript. WY revised the manuscript. ZZ and WY confirmed the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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